An examination of the basic blue copper protein from cucumber peelings by ^{1}H-NMR

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The ^{1}H-NMR spectrum of cucumber basic blue protein (CBP) has been recorded. Examination of the spectrum of the reduced protein suggests that one or more sidechains exist in conformations which interconvert slowly at ambient temperatures. His 39, His 84 and Met 89 are identified as copper ligands by redox titration and by amino acid sequence homology with plastocyanin and azurin. The importance of a Phe sidechain close to the Met ligand in the high potential blue copper site is confirmed. Broadening of His ligand resonances at elevated temperatures reveals an exchange process at the reduced copper centre.

Cucumber basic blue protein *^{1}H-NMR* *Type I copper* *Copper ligand* *Histidine*

1. INTRODUCTION

'Type I' [1] or 'blue' copper centres in proteins are characterized by the presence of an intense electronic absorption near 600 nm, a small hyperfine splitting constant $A_\parallel$ in the EPR spectrum and a high redox potential [2]. Proteins with a single type I copper centre include plastocyanin, azurin, stellacyanin, a basic blue protein from cucumber and others. The crystal structure analyses of poplar plastocyanin [3–5] and two azurins [6–8] have revealed the structural features responsible for the distinctive physical properties of the blue copper centre. The copper sites of plastocyanin and azurin possess an irregular tetrahedral coordination geometry, with two nitrogen and two sulfur ligands supplied by the side-chains of two His, one Cys and one Met residue. However, the amino acid sequence of stellacyanin [9] contains no Met, and a different coordination, involving a second Cys [10] or a disulfide group [11] as fourth ligand, has been proposed. It is apparent that subclasses of blue copper proteins must exist. A tentative classification scheme has been recently suggested [12].

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The basic blue copper protein isolated from cucumber peelings [13,14] and cucumber seedlings [15,16] has physical properties that make its classification difficult. Authors in [17] proposed that the name 'cucumber basic blue protein' (CBP) be retained for this protein, in preference to the earlier names 'cusacyanin' and 'plantacyanin' [13,14], until its function is established. The spectroscopic properties of CBP resemble those of stellacyanin [14], but the redox potential is closer to that of azurin. The amino acid sequence of CBP from seedlings [17] contains 3 half-Cys and two Met residues that are potential ligands.

Here we identify 3 of the copper ligands of CBP on the basis of ^{1}H-NMR experiments on protein from cucumber peelings. Assuming that the basic blue proteins from cucumber seedlings and peelings are identical, we discuss our results in terms of residue numbers derived from the amino acid sequence of the seedling protein.

2. MATERIALS AND METHODS

CBP (2.5 mg) was isolated from cucumber peelings by a slight modification of the method in [15]. The purified protein ran as a single band on PAGE and exhibited an A_{280}/A_{597} ratio of less than 6.

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Solutions for NMR were prepared by exchanging concentrated protein into deuterated solvent by dialysis.

NMR spectra were recorded on a Bruker WM-400 spectrometer. Resolution enhancement was performed by Gaussian transformation. The Carr-Purcell method A pulse sequence was used for multiplet selection [18]. Chemical shifts are referred to internal dioxane at 3.750 ppm. Quoted pH values are uncorrected meter readings.

3. RESULTS

Fig.1 shows the NMR spectrum of reduced CBP. The spectrum resembles those of plastocyanin [19,20], azurin [19] and stellacyanin [10] in its general features. Resonances of many slowly exchanging amide protons are evident between 7.5 and 10.5 ppm and several ring-current shifted methyl group resonances occur upfield of 0.5 ppm. The protein is therefore tightly folded. One unusual feature is the presence of signals at about 25% intensity at the sides of major peaks in the 0.5 to −1.0 ppm region (fig.1a,2a). These signals may be due to microheterogeneity of the amino acid sequence or slowly interconverting conformations. Amino acid sequence data indicate no heterogeneity in CBP from seedlings [17], so it is unlikely to occur for protein from peelings. We conclude that there is a slow exchange between two or more quite similar conformations, such as may arise from different orientations of a Trp sidechain. There is no evidence that the additional resonances are due to denaturation.

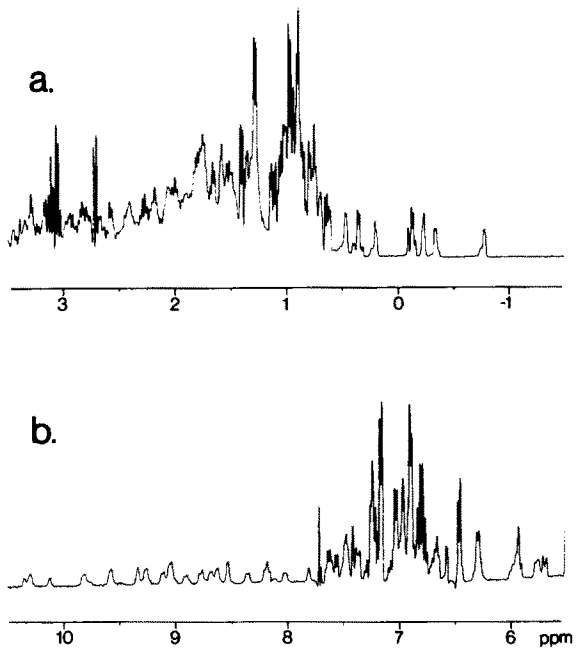

Fig.1. ^{1}H-NMR spectrum of 0.1 mM reduced CBP in 20 mM phosphate buffer (pH 7.5), 298 K. (a) Upfield region including ring current shifted methyl signals. (b) Downfield region including unexchanged amide signals (× 2).

3.1. *Resonance assignment*

CBP contains two His (39 and 84) and two Trp (11 and 18) residues [17] which are expected to produce six 1-proton singlets in the low-field region of the spectrum (fig.1b). Two clearly resolved singlets at 7.71 and 7.41 ppm are assigned to His C-2 or C-4 protons on the basis of their chemical shifts. The Carr-Purcell method A experiment reveals two further singlets at 7.21 and 7.15 ppm which are tentatively assigned to His. The two remaining singlets have not been located with certainty. There is however a resolved peak at 6.56 ppm (fig.1b) which may be a Trp C-2 proton resonance showing the effects of conformational heterogeneity. It is an apparent doublet with a barely resolvable splitting at 298 K, and two separated singlets of differing intensities at higher temperatures.

The resonances assigned to His above do not shift on changing the pH from 7.5 to 6.5. The absence of free titration is consistent with a role for both His 39 and His 84 as copper ligands.

CBP contains two Met (38 and 89) residues [17]. One singlet resonance (M1) is clearly resolved at −0.09 ppm (fig.1a). A singlet in this region of the spectrum can only be a strongly ring current shifted ϵ-methyl resonance from Met. However, M1 is only 1.5 protons in intensity. A second singlet resonance (M2), overlapping with another signal at −0.23 ppm, is revealed in Carr-Purcell method A spectra. The intensity of this resonance cannot be measured accurately, but certainly corresponds to less than 3 protons. It is likely that resonances M1 and M2 arise from the ϵ-methyl group of a single Met in two different conformational substates. The ϵ-methyl resonance of the other Met residue is not resolved in Carr-Purcell method A spectra.

3.2. *Redox titration*

Fig.2 shows the results of a titration of the protein with ferricyanide. Partial oxidation produces specific broadening of resonances from protons close to the copper. The Met singlet M1 and the His singlets H1-4 (fig.2a) are prominent in a difference spectrum between reduced and 2% oxidized protein (fig.2b). The other singlet resonance M2 also appears in the difference spectrum, overlapping with another methyl signal. Other resonances appear in the difference spectrum as the percentage oxidation increases. His 39 and 84 and a Met residue are therefore close to the copper, and may be assumed to be ligands by analogy with plastocyanin and azurin. The fact that the partial resonances M1 and M2 both appear in the difference spectrum supports their assignment to the same Met residue.

3.3. *Temperature dependence*

Increasing temperature has a marked effect on the spectrum of reduced CBP. Fig.3 shows that the resonances H2 and H4 (arrowed) broaden on increasing the temperature from 298 to 313 K. Resonances M1 and M2 appear to be unaffected. One or more nearby methyl group resonances shift downfield to overlap with M1, but M1 itself does not seem to shift or broaden. Similarly, the other methyl group resonance overlapping with M2 shifts fractionally. The broadening of the His signals indicates that there are exchange processes occurring at the copper site.

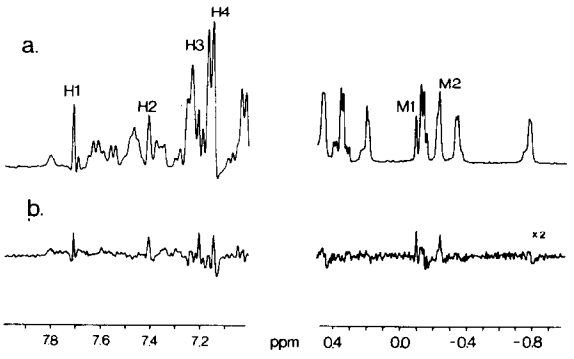

Fig.2. Redox titration with ferricyanide (pH 7.5), 298 K. (a) Normal spectrum of reduced CBP. (b) Difference spectrum of reduced −2% oxidized protein. Assigned resonances are indicated.

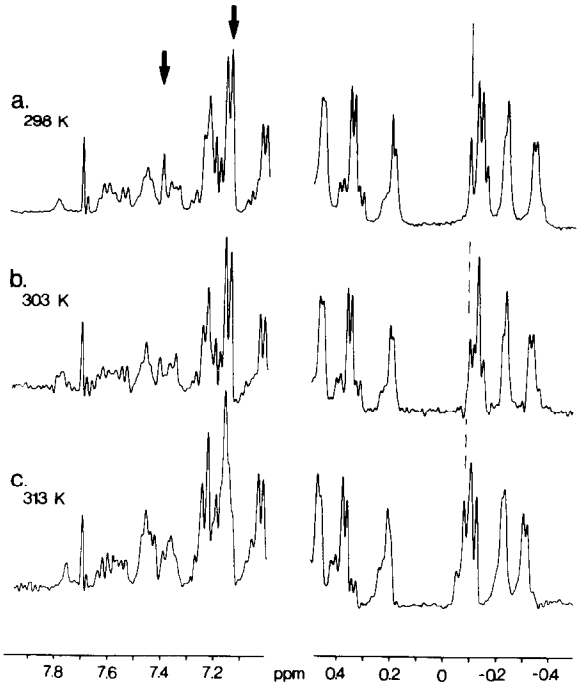

Fig.3. Temperature dependence of NMR spectrum of reduced CBP. Arrowed resonances broaden on increasing temperature.

4. DISCUSSION

We conclude from the above experiments that CBP possesses two His and one Met ligand. It is presumed that the fourth ligand is a Cys residue as the intense colour of blue copper proteins has been firmly ascribed to Cys⟶Cu charge transfer [21]. From amino acid sequence homology [17], it is likely that Met 89 is the Met ligand involved in CBP. In this event, CBP possesses the plastocyanin/azurin coordination type rather than that of stellacyanin. Table 1 compares the 'structure shifts' (see legend for definition) for the ligand resonances of plastocyanin, CBP and azurin. The structure shifts for CBP and plastocyanin are remarkably similar if specific His assignments for CBP are assumed to be as shown. This is despite the fact that the amino acid sequence of CBP is closer to that of stellacyanin than that of plastocyanin.

The large upfield shift experienced by the ϵ-methyl resonance of the ligand Met 92/121 in plastocyanin/azurin is due primarily to a ring cur-

Table 1
Comparison of structure shifts for ligand resonances of plastocyanin (Pc), CBP and azurin (Az)

Residue (Pc, CBP, Az)		Structure shifts[a]		
		Pc[b]	CBP[c]	Azurin[c]
His (87,84,117)	C-2	−0.05 [−0.09]	(−0.03)	(−0.82)
	C-4	0.05 [0.03]	(0.18)	(−0.08)
His (37,39,46)	C-2	−0.55 [−0.61]	(−0.54)	(−0.88)
	C-4	0.63 [0.64]	(0.44)	(−0.17)
Met (92,89,121)	ε-Me	−1.55 [−1.61]	−2.22/−2.36	−2.18/−2.02

[a] δexp − δrandom coil [22]
[b] Shifts for French bean and [poplar] plastocyanins (G. King and P.E. Wright, unpublished)
[c] Shifts in round brackets are based on tentative assignments only, following the assignment scheme for plastocyanin. Azurin data taken from [23,24]

rent from the conserved residue, Phe 14/15. By homology, the large shift on Met 89 in CBP is probable due to Phe 13. A shift of this magnitude can occur only if the methyl group is in Van der Waals contact with the aromatic ring. The presence of this effect in CBP supports the suggestion [5] that this aromatic sidechain is an essential component of the blue copper site.

The nature of the conformational substates of CBP and the origin of the exchange processes at the reduced copper site are unclear. Conformational heterogeneity and exchange processes affecting the Met ligand have also been reported for azurin [23,24].

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